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Padma Baskar
Art Unit 1645
Patent Examiner/Biotechnology
CM-1, 8E-13
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THE PURIFICATION AND CHARACTERIZATION OF AN ANTIHEMORRHAGIC FACTOR IN OPOSSUM (*DIDELPHIS VIRGINIANA*) SERUM

JUAN M. MENCHACA and JOHN C. PEREZ
Department of Biology, Texas A & I University, Kingsville, Texas 78363, U.S.A.

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JUAN M. MENCHACA and JOHN C. PEREZ. The purification and characterization of an antihemorrhagic factor in opossum (*Didelphis virginiana*) serum. *Toxicon* 19, 623–632, 1981.—Antihemorrhagic protein isolated from serum of the opossum (*Didelphis virginiana*) was homogeneous by polyacrylamide disc electrophoresis. The purified antihemorrhagic factor had an isoelectric pH of 4.1, a molecular weight of 68,000, migrated as the fastest band in disc electrophoresis, failed to form a precipitate with crude *Crotalus atrox* venom, and did not show proteolytic activity on gelatin. This evidence indicates that the antihemorrhagic factor in the serum of the opossum is albumin or closely associated with albumin.

INTRODUCTION

PREVIOUS studies have shown that serum of some snakes neutralizes the effects of snake venoms. Both OMORI-SATOH *et al.* (1972) and OVADIA (1978) have purified and characterized antihemorrhagic factors in the serum of snakes. Certain warm-blooded animals have shown a remarkable resistance to toxic action of snake venoms (KILMON, 1976; OVADIA and KOCHVA, 1977; WERNER and VICK, 1977; PEREZ *et al.* 1978 *a* & *b*). PEREZ *et al.* (1978*b*) reported that 16 of 40 species of warm-blooded animals that were tested had a serum antihemorrhagic titer of 4 or greater against *Crotalus atrox* venom. PICHYANGKUL and PEREZ (1981) isolated and characterized an antihemorrhagic factor in the serum of *Sigmodon hispidus* which had physical properties different from immunoglobulins.

The Virginia opossum (*Didelphis virginiana*) is one of the largest and most available resistant warm-blooded animals in Texas. The opossum's resistance to crotalid venoms was first reported by KILMON (1976) and then by WERNER and VICK (1977). PEREZ *et al.* (1978*b*) reported that the hemorrhagic activity of *Crotalus atrox* venom was neutralized with opossum serum. A knowledge of the mechanism of venom neutralization in resistant animals may bring about a better understanding of the toxic nature of snake venom and of venom neutralization. Such understanding may also further the development of better methodology in snakebite treatment and in other hemorrhagic diseases. The present study undertook the purification and characterization of the antihemorrhagic factor in serum of the opossum as prerequisite to study of the venom neutralization mechanism.

MATERIALS AND METHODS

Materials

Sephadex G-200, G-75 superfine, G-100, and DEAE A-50 were purchased from Pharmacia Fine Chemicals. LKB ampholytes were purchased from LKB-Produkter. Amid Black and N,N' Methylene-Bisacrylamide (BIS) were purchased from the Ames Company; Bromo-phenol blue, tetramethylethylenediamine, riboflavin, ammonium

persulfate, and polyacrylamide from E–C Apparatus Corporation; NaCl, biuret reagent, and Tris (hydroxymethyl) aminoethane from the Fisher Scientific Company. All other chemicals utilized were of the highest purity. New Zealand white rabbits were purchased locally. Opossum serum was acquired from opossums trapped in Kleberg Co., Texas. Opossums were bled by heart puncture, after which the serum was separated by centrifugation at 650 *g* for 10 min and stored at −90°C. Pooled serum was thawed and lyophilized in 5-ml portions and again stored at −90°C until used. Venom from *Crotalus atrox* was collected at the annual Freer, Texas, Rattlesnake Roundup. The venom was cooled to 4°C, centrifuged to remove cellular debris, pooled, lyophilized and stored at −90°C until used.

Protein estimation and concentration of protein

The protein content of venom, serum, and serum fractions was determined by the Biuret assay using bovine serum albumin (V) as standard. The Beckman Model 25 spectrophotometer was standardized with a biuret solution (4 ml biuret + 1 ml saline) at 540 nm, and the optical density of the sample dilutions was measured (GORNALL *et al.* 1949). Protein solutions were concentrated by lyophilizing under vacuum in a VirTis lyophilizer.

Hemorrhagic assay

A hemorrhagic assay previously described, OMORI-SATOH *et al.* (1972), was used to establish a minimal hemorrhagic dose for the venom by serially diluting the venom and injecting 0.1 ml intracutaneously into the depilated back of rabbits. The rabbits were sacrificed 24 hr later and the skins removed. The minimal hemorrhagic dose (2.6 μg) is defined as that concentration of venom resulting in a 10 mm hemorrhagic spot.

Antihemorrhagic assay

Equal volumes of minimal hemorrhagic dose of the venom and doubling dilutions of opossum serum were incubated for 1 hr at 25°C. The back of a rabbit was depilated and 0.1 ml of each of the incubated mixtures was injected intracutaneously. The rabbits were sacrificed after 24 hr and the skins removed. The reciprocal of the highest dilution of serum blocking the minimal hemorrhagic dose was taken as the titer, also referred to as activity.

Proteolytic activity

Proteolytic activity of the antihemorrhagic factor was tested on X-ray film coated with gelatin. Ten micrograms of crude opossum serum and purified antihemorrhagic factor were applied to X-ray film and incubated for 20 hr at 37°C in a moist chamber. *C. atrox* venom and 0.1 M Tris–HCl buffer, pH 8.1, were used as controls. The film was soaked in deionized water for 5 min and then gently wiped with a wet sponge. Gelatin digestion was indicated by a transparent spot on the film.

Thermostability and pH stability

Thermostability of the antihemorrhagic activity in crude serum was examined. Opossum serum was heated for 30 min at various temperatures (25, 37, 55, 75 and 100°C). After cooling to room temperature, each sample was assayed for antihemorrhagic activity.

The stability of antihemorrhagic activity in the crude serum at various pH values was examined. Two-fold serial dilutions were made of crude serum with phosphate buffer of varying pH (2–12). The solutions were kept at 4°C for 24 hr. The pH was readjusted to pH 7.5 by dialyzing in 7.5 pH phosphate buffer for 24 hr. Each dilution was assayed for antihemorrhagic activity.

Disc electrophoresis

The purity of the antihemorrhagic factor was checked by disc electrophoresis (DAVIS, 1964), using a disc electrophoresis Buchler Polyanalyst. Various concentrations of whole serum and the purified antihemorrhagic factor were added to 0.02% Bromo-phenol Blue in 20% sucrose. Samples containing 700 μg of whole serum, 300 μg of peak C from the G-200 process, 200 μg of peak F from the DEAE Sephadex (A-50) process and 100 μg of peak G from the G-100 process were applied to polyacrylamide gels. The gels were in contact with Tris–glycine buffer, pH 8.9, at both ends. An electric current of 4 mA per tube (gel column size 5 × 75 mm) was applied. The run was terminated when the dye reached within a few mm from the end of the gel column. The gel was stained with a 2–5% solution of Amido Black in acetic acid, methanol, and water solution in proportions 1/3/6 respectively. Destaining was done over a 24 hr period with a solution of 1 part acetic acid, 3 parts methanol and 6 parts water.

Molecular weight determination

The molecular weight of the antihemorrhagic factor was determined by the method of ANDREWS (1964). Four known molecular weight proteins were used as standards. Five hundred micrograms of horse heart myoglobin (mol. wt 16,900), 500 μg of carbonic anhydrase (mol. wt 29,000), 1.0 mg of human albumin (mol. wt 68,000), 1.0 mg of transferrin (mol. wt 77,000), and 1.1 mg of purified antihemorrhagic factor were applied separately to a K9/30 column of Sephadex G-200. In each case the column was equilibrated with 0.1 M Tris–HCl, pH 8.1. This same buffer was used as the eluant. One-milliliter fractions were collected with a flow rate of 0.055 ml/min and a head pressure of 23 cm of water. The relative protein concentration of each fraction was read with a Beckman Model 25 spectrophotometer at 280 nm. The logarithm of each known molecular weight was plotted against the elution volume. Once the elution volume was determined for the antihemorrhagic factor, the molecular weight of the antihemorrhagic factor was estimated from the standard curve.

Gel filtration on Sephadex G-200

Twenty-five milliliters of whole opossum serum, containing 1.7 f total protein, were applied t a K50/100 column with a bed volume of 1.8 l. The column had been previously filled with swollen G-200 Sephadex beads and equilibrated with 0.1 M Tris–HCl buffer, pH 8.1. This same buffer was used t elute the sample at a constant flow rate of 0.4 ml/min with a constant head pressure of 11 cm of water. Seventy fractions, each 15 ml, were collected. Their relative protein concentrations were determined using spectrophotometry. The absorbance of each fraction was plotted against the fraction number. Each fraction was also assayed for antihemorrhagic activity. The absolute protein concentration of the active fractions was determined by the biuret method at 540 nm. The specific activity, (activity per total amount of protein), was also determined for the active fractions and is an indication of relative purities.

Ion exchange chromatography on DEAE A-50

The second step in the isolation process was to take the active samples from the gel filtration process and to subject them to ion exchange chromatography on DEAE A-50 Sephadex.

A K9/30 column was filled with 19 ml of preswollen DEAE A-50 Sephadex beads. The column was equilibrated with 0.025 M Tris–HCl at a pH of 8.1. Ten milliliters of an active sample from the G-200 column, containing 25.7 mg of protein, were introduced into the column. The sample was eluted with three different buffers with a constant head pressure of 20 cm of water. Tris–HCl buffer, 0.025 M, was used to collect the first 15 fractions; Tris–HCl buffer, 0.025 M and 0.1 N NaCl, was used to elute the next 68 fractions; Tris–HCl buffer, 0.1 M and 0.3 N NaCl, was used for the last 37 fractions. The initial flow rate was 0.056 ml/min but increased to 0.28 ml/min when high salt was used. The relative protein concentration and antihemorrhagic activity were measured for each of the 120 one ml fractions from the A-50 Sephadex column. The absorbance of each fraction was plotted against the fraction number. The absolute protein concentration of the composite active peaks was determined by the biuret method at 540 nm. The specific activity for the composite was also determined.

Gel filtration on Sephadex G-100

The active samples from the DEAE A-50 run were pooled, lyophilized, and reconstituted to a volume of 1 ml (6.63 mg of total protein). This sample was applied to a K9/30 column of G-100 Sephadex. The gel was equilibrated with 0.1 M Tris–HCl buffer, pH 8.1. The same buffer was used to elute the sample at a constant flow rate of 0.5 ml/min with a head pressure of 36 cm of water. Fifty 1-ml fractions were collected, and their relative protein concentrations were measured at 280 nm. The antihemorrhagic activity was measured to determine the location of the active fraction. The active samples were pooled, and the specific activity was determined.

Isoelectric focusing

The isoelectric pH of the antihemorrhagic factor was determined using the method of WINTER *et al.* (1975) with LKB 2117 Multiphor. The flat-bed electrofocusing was conducted in Sephadex G-75 superfine gel with the carrier ampholytes covering the pH range from 3.5 to 10. A composite of the active samples from the G-100 process, a total of 1.32 mg of protein, was applied as a sample zone 10 cm from the anode. The electrofocusing was conducted for 7 hr at 4°C with an initial power of 4.5 W and a final power of 27 W. When the separation was complete a print was taken of the entire bed by placing a piece of dry filter paper on the gel surface for 2 min. The print was removed and dried in an oven. Then it was washed three times for 15 min in 10% TCA, stained with 0.2% Coomassie brilliant blue R 250 dissolved in methanol/water/acetic acid in the proportions 5/5/1, and destained with frequent changes of the methanol/water/acetic acid solution. The bed was divided into 30 compartments by using a 22 × 10.25 mm grid. The pH of each fraction was taken using a surface pH electrode. Five fractions of the gel were scooped up and pooled separately. Each fraction consisted of a varying number of compartments: the first included Nos. 1–13, the second Nos. 14–19, the third Nos. 20–23, the fourth Nos. 24–26, and the fifth Nos. 27–30. Ten milliliter syringes were used as columns for elution of protein from the gels. One milliliter of 0.85% saline was used per compartment to elute each fraction. The eluate from each column was dialyzed in 0.85% saline for 24 hr. After dialysis the relative protein concentration of each eluate was determined with the spectrophotometer at 280 nm. The eluates were lyophilized and reconstituted to a concentration of 1.45 mg/ml. They were then assayed for antihemorrhagic activity.

RESULTS

A summary of the purification of the antihemorrhagic factor from 1.7 g of crude opossum serum is presented in Table 1. All steps were performed at 4°C, unless otherwise indicated.

Purification

The results of the gel filtrati n step n Sephadex G-200 are presented in Fig. 1. Three definite peaks were eluted from the gel filtration column. The solid line represents the relative pr tein c ncentrati n, and the shaded area indicates antihemorrhagic activity. The

TABLE 1. PURIFICATION OF THE ANTIHEMORRHAGIC FACTOR FROM THE SERUM OF THE POSSUM (*Didelphis virginiana*)

Step	Concentration* (mg/ml)	Activity†	Specific activity‡	Purification factor§
Crude serum	68	128	37.6	1
G-200 Sephadex peak C	2.4	8	66.1	1.7
DEAE A-50 Sephadex peak F	2.2	16	144.8	3.8
G-100 Sephadex peak G	1.3	16	242.4	6.4

* The protein concentration was measured by biuret assay at 540 nm. BSA was used as standard protein.

† The activity (sometimes referred to as titer) is expressed as the reciprocal of the highest dilution of a sample blocking the minimal hemorrhagic dose (MHD) of venom.

‡ The specific activity is expressed as the activity divided by the total protein (mg) in each sample.

§ The purification factor is the number of times that specific activity increased over the crude serum.

antihemorrhagic activity, associated only with peak C, was increased 1.7 times over that of the original crude serum, as shown in Table 1.

DEAE ion exchange chromatography was used to further fractionate peak C from the Sephadex G-200 column into 3 peaks. Two peaks, D and E, were eluted with 0.025 M Tris–HCl buffer and 0.1 N NaCl at pH 8.1. Peak D and E were separately pooled and concentrated, by lyophilization, to four times their original concentrations. The concentrated

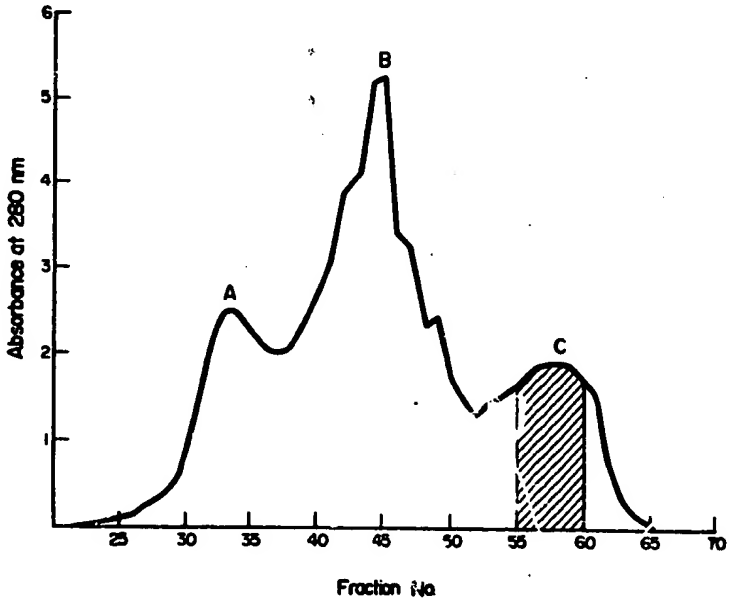

FIG. 1. GEL FILTRATION ON G-200 SEPHADEX.
Crude opossum serum containing 1.7 g f protein in 25 ml was applied t a K50/100 column as detailed in "Purification procedures". The shaded area under peak C shows the location of the antihemorrhagic activity.

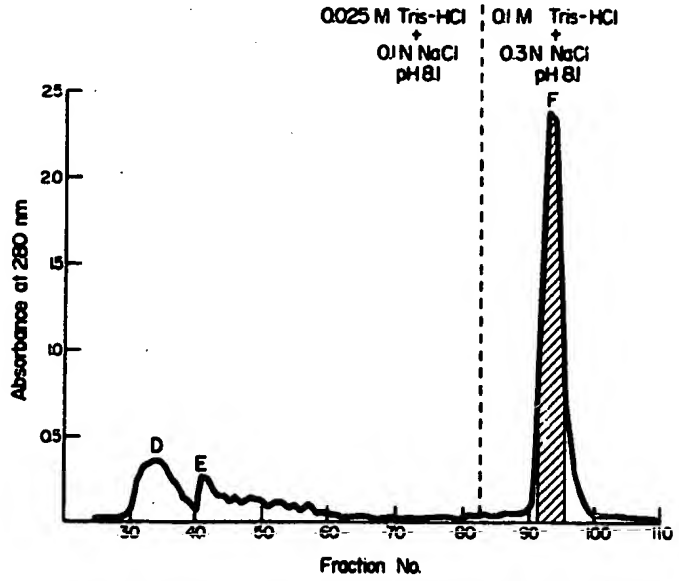

FIG. 2. ION-EXCHANGE CHROMATOGRAPHY ON DEAE A-50 SEPHADEX.
A total of 25.7 mg of protein from peak C from the G-200 gel filtration process was applied to a K9/30 column as detailed in "Purification procedures". The shaded area under peak F shows the location of the antihemorrhagic activity.

peaks exhibited no antihemorrhagic activity. An additional peak, F, was eluted with 0.1 M Tris–HCl and 0.3 N NaCl at pH 8.1. The solid line in Fig. 2 represents the relative protein concentration, and the shaded area under peak F indicates the location of the antihemorrhagic activity. The specific antihemorrhagic activity of a composite of the active samples was increased 3.8 times over that of the original crude serum, as shown in Table 1.

Further fractionation of peak F from the ion exchange column on Sephadex G-100 resulted in the elution of 2 peaks (Fig. 3). Peak G consisted of samples Nos. 6–11, and peak H,

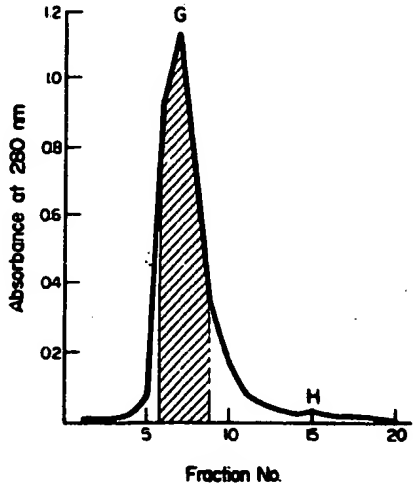

FIG. 3. GEL FILTRATION ON G-100 SEPHADEX.
A total of 6.60 mg of protein from peak F of the DEAE A-50 process was applied to a K9/30 column packed with G-100 Sephadex as detailed in "Purification procedures". The shaded area under peak G represents the location of the active fractions.

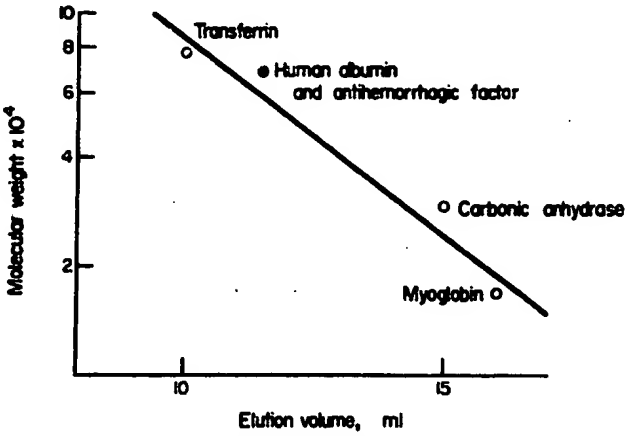

FIG. 5. DETERMINATION OF THE MOLECULAR WEIGHT OF THE ANTIHEMORRHAGIC FACTOR. Known molecular weight proteins were run separately in a K9/30 column of G-200 Sephadex as detailed in "Analytical methods". The logarithm of each known molecular weight was plotted against the elution volume. The antihemorrhagic factor was run under the same conditions, and the molecular weight was taken from the standard curve.

being much smaller, was only sample 15. The antihemorrhagic activity is represented as the shaded area and was associated with peak G. The specific antihemorrhagic activity of peak G was increased 6.40 times over that of crude serum (Table 1).

Purity

The results of polyacrylamide disc electrophoresis are presented in Fig. 4. Gel A, crude serum, had many bands throughout the entire length of the column. Gel B, a composite of the active samples from the G-200 gel filtration step, showed a lack of the γ globulin which was present in crude serum. Gels C and D were homogeneous with one fast migrating band in the area of albumin.

Proteolytic activity

Proteolytic activity of the antihemorrhagic factor was tested on X-ray film coated with gelatin. Ten micrograms of *C. atrox* venom, crude opossum serum, purified antihemorrhagic factor, and 0.1 M Tris–HCl pH 8.1 were applied to X-ray film and incubated for 10 hr at 37°C in a moist chamber. After soaking the film in deionized water and wiping with a sponge, only the venom control exhibited gelatinase activity. Neither crude serum nor purified factor had gelatinase activity.

Thermostability

Thermostability of the antihemorrhagic activity in crude opossum serum was examined by heating the serum at various temperatures for 30 min. Antihemorrhagic activity was stable at temperatures of 0–37°C.

pH stability

The stability of the antihemorrhagic activity in the crude serum at various pH values was examined. Two-fold serial dilutions of crude serum were made with phosphate buffer with various pH ranging from 2 to 12. Antihemorrhagic activity was stable from pH 3 t 10.

Molecular weight determination

The molecular weight of the antihemorrhagic factor was determined by gel filtration; f ur

FIG. 4. DISC ELECTROPHORETIC PATTERNS OF CRUDE SERUM AND THE PURIFIED FRACTIONS AS DETAILED UNDER "ANALYTICAL METHODS".

Gels A, B, C and D respectively correspond to whole serum (700 μg), peak C from the G-200 process (300 μg), peak F from the DEAE A-50 process (200 μg) and peak G from the G-100 process (100 μg).

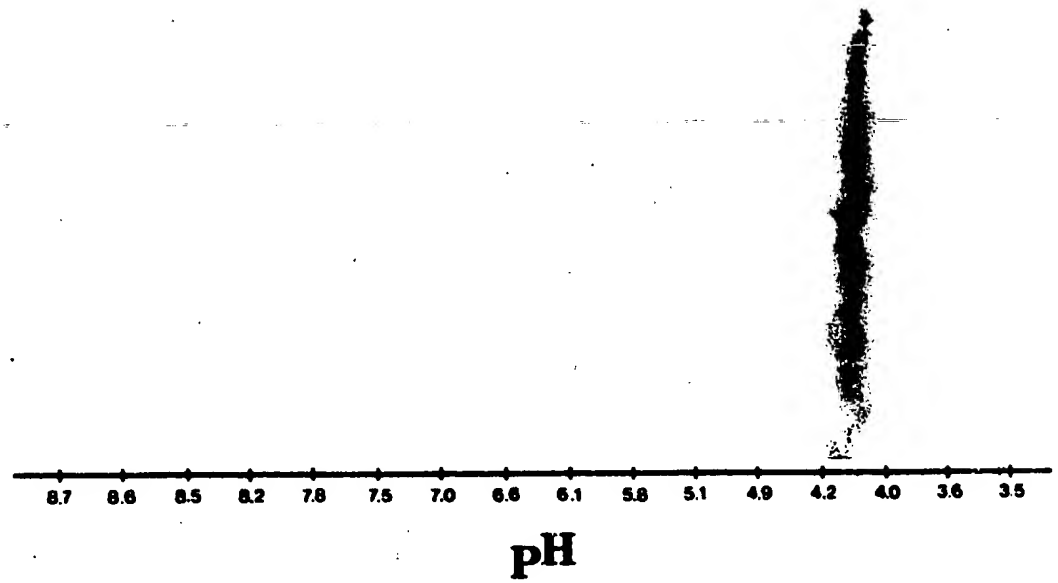

FIG. 6. PREPARATIVE FLAT-BED ELECTROFOCUSING OF THE ANTIHEMORRHAGIC FACTOR FROM THE G-100 GEL FILTRATION PROCESS.

One milliliter of peak G, containing 1.32 mg of protein, was applied to the process as detailed in "Purification procedures". The pH gradient was measured and ranged from 3.5 to 8.7. The print showed an intense band between pH 4.0 and 4.2 which lost its antihemorrhagic activity.

TABLE 2. A COMPARISON F ISOLATED ANTIHEMORRHAGIC FACT RS

Characteristics	a	b	c	d
Thermostability	0–56°	0–85°	0–55°	0–37°
pH Stability	2–11	4–9.5	3–10	3–10
Molecular weight	70,000	80,000	90,000	68,000
Isoelectric pH	4.0	4.7	5.4	4.1
Proteolytic activity		no	no	no
Ring precipitin test	no ppt line	no ppt line	no ppt line	no ppt line

a *Trimeresurus flavoviridis* (OMORI-SATOH *et al.*, 1972).
b *Vipera palaestinae* (OVADIA, 1978).
c *Sigmodon hispidus* (PICHYANGKUL and PEREZ, 1981).
d *Didelphis virginiana* (MENCHACA and PEREZ, present study).

known molecular weight proteins were run separately on a K9/30 column of Sephadex G-200. The logarithm of each known molecular weight was plotted against elution volume of human albumin (Fig. 5). The molecular weight of the antihemorrhagic factor was estimated to be 68,000.

Isoelectric focusing

The paper print of the gel bed revealed one intense band between pH 4.0 and 4.2 (Fig. 6). The gel bed was divided into 5 fractions: fraction 1 extended from pH 6.9 to 8.7, fraction 2 from 5.3 to 6.6, fraction 3 from 4.8 to 5.1, fraction 4 from 4.0 to 4.2, and fraction 5 from 3.5 to 3.9. The protein was eluted from each section and concentrated by lyophilization to 1.45 mg/ml; however, no antihemorrhagic activity was detected in any of the fractions. The antihemorrhagic factor appears to have lost its activity during the isoelectric focusing step.

DISCUSSION

A sequence of techniques was used to purify and characterize the antihemorrhagic factor from the serum of the opossum. The G-200 Sephadex gel filtration process revealed three distinct peaks. This is consistent with previous work of ROWLANDS and DUDLEY (1968) in which they studied the immunoglobulins in adult opossums. Disc electrophoresis of the active fractions from the ion exchange and G-100 gel filtration processes showed the samples to be homogeneous. Most techniques used increased the specific activity of the antihemorrhagic factor, with the exception of flat-bed electrofocusing after which all activity was lost. OVADIA (1978) also reported some loss of antihemorrhagic activity for the purified factor in *Vipera palaestinae* serum after an electrofocusing step. The paper print revealed one band, but hemorrhagic activity was lost.

Table 2 compares the characteristics of the opossum antihemorrhagic factor to the antihemorrhagic factor from *Trimeresurus flavoviridis* (OMORI-SATOH *et al.*, 1972), *Vipera palaestinae* (OVADIA, 1978) and *Sigmodon hispidus* (PICHYANGKUL and PEREZ, 1981).

The opossum antihemorrhagic factor was less stable at higher temperatures than the others. The pH stability of the opossum antihemorrhagic factor at extreme values was similar to the other factors. The molecular weight of the opossum antihemorrhagic fact r was 68,000, smaller than *T. flavoviridis* antihem rrhagic factor (70,000), *V. palaestinae* antihemorrhagic fact r (80,000) and *S. hispidus* antihemorrhagic factor (90,000). The isoelectric pH f the opossum antihem rrhagic was 4.1; this m re closely resembles the isoelectric pH f the antihemorrhagic factor purified from the serum f *T. flavoviridis*.

The isoelectric pH, 4.1, and molecular weight, 68,000, are very similar t those of albumin. The fact that the purified possum antihemorrhagic factor appears as the fastest migrating band in disc electr phoresis also leads us to believe that the fact r is either albumin r is closely associated with it. If the antihemorrhagic factor and albumin are separate proteins, the methods used in this study were not able to separate them from each other.

The mechanism by which this factor neutralizes the hemorrhagic effects of *C. atrox* venom is unclear; however, the ring precipitation test failed to show precipitin formation with purified antihemorrhagic factor and crude rattlesnake venom. Similar results have been shown with the purified antihemorrhagic factors from *T. flavoviridis, V. palaestinae* and *S. hispidus*. This suggests that the antihemorrhagic factor in sera of animals having a natural resistance to the effects of snake venoms does not form a complex similar to that formed by the classic antibody-antigen reaction. Perhaps a more definite conclusion can be drawn if homogeneous hemorrhagic toxins, such as those previously isolated from the venom of *C. atrox* (BJARNASON and TU, 1978), were used instead of crude venom. Failure of the purified hemorrhagic factor to show proteolytic activity suggests that the mechanism of neutralization is not enzymatic in nature, unless the antihemorrhagic factor has a highly specific substrate.

Whatever the mechanism, the opossum purified antihemorrhagic factor neutralizes the crude *C. atrox* venom which contains five hemorrhagic toxins (BJARNASON and TU, 1978). PEREZ *et al.* (1978*b*) previously reported that crude opossum serum neutralized the hemorrhagic factors in crude venom. Better understanding of the mechanism of neutralization in resistant warm-blooded animals will increase our understanding of venom neutralization in other animals.

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